Pharmacologic Targeting of Bacterial β-Glucuronidase Alleviates Nonsteroidal Anti-Inflammatory Drug-Induced Enteropathy in Mice

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ABSTRACT

Small intestinal mucosal injury is a frequent adverse effect caused by nonsteroidal anti-inflammatory drugs (NSAIDs). The underlying mechanisms are not completely understood, but topical (luminal) effects have been implicated. Many carboxylic acid-containing NSAIDs, including diclofenac (DCF), are metabolized to acyl glucuronides (AGs), and/or ether glucuronides after ring hydroxylation, and exported into the biliary tree. In the gut, these conjugates are cleaved by bacterial β-glucuronidase, releasing the potentially harmful aglycone. We first confirmed that DCF-AG was an excellent substrate for purified *Escherichia coli* β-D-glucuronidase. Using a previously characterized novel bacteria-specific β-glucuronidase inhibitor (Inhibitor-1), we then found that the enzymatic hydrolysis of DCF-AG in vitro was inhibited concentration dependently (IC$_{50}$ ∼164 nM). We next hypothesized that pharmacologic inhibition of bacterial β-glucuronidase would reduce exposure of enterocytes to the aglycone and, as a result, alleviate enteropathy. C57BL/6J mice were administered an ulcerogenic dose of DCF (60 mg/kg i.p.) with or without oral pretreatment with Inhibitor-1 (10 μg per mouse, b.i.d.). Whereas DCF alone caused the formation of numerous large ulcers in the distal parts of the small intestine and increased (2-fold) the intestinal permeability to fluorescein isothiocyanate-dextran, Inhibitor-1 cotreatment significantly alleviated mucosal injury and reduced all parameters of enteropathy. Pharmacokinetic profiling of DCF plasma levels in mice revealed that Inhibitor-1 coadministration did not significantly alter the C_{max}, half-life, or area under the plasma concentration versus time curve of DCF. Thus, highly selective pharmacologic targeting of luminal bacterial β-D-glucuronidase by a novel class of small-molecule inhibitors protects against DCF-induced enteropathy without altering systemic drug exposure.

Introduction

Gastrointestinal (GI) injury is one of the major adverse effects associated with nonsteroidal anti-inflammatory drugs (NSAIDs). This iatrogenic disease is manifested as ulceration and bleeding of the mucosa, inflammation, and even perforation (Allison et al., 1992; Bjarnason et al., 1993; Wolfe et al., 1999). With the advent of novel diagnostic tools, including video capsule endoscopy, it has become increasingly clear that not only the stomach, but also the small intestine, is a major target of NSAID-associated toxicity (Davies et al., 2000; Koga et al., 2008; Scarpignato and Hunt, 2010). Indeed, approximately two-thirds of both long-term (>3 months) and short-term (∼1 week) NSAID users exhibited mild or more severe forms of drug-induced lesions in the small intestine (Fortun and Hawkey, 2007; Maiden, 2009). In addition, many unexplained GI lesions in "control subjects" were found to be attributable to nonprescription use of NSAIDs (Sidhu et al., 2010). Despite this high incidence of the disease, there are currently no approved therapies to prevent or treat NSAID enteropathy.

Part of the reasons for a lack of therapies is an incomplete understanding of the underlying mechanisms (Whittle, 2004). The mode of toxicity to the small intestinal mucosa is

ABBREVIATIONS: GI, gastrointestinal; DCF, diclofenac; DCF-AG, diclofenac-1-β-O-acyl glucuronide; FITC, fluorescein isothiocyanate; Inh-1, Inhibitor-1 [1-((6,8-dimethyl-2-oxo-1,2-dihydroquinolin-3-yl)methyl)-3-(4-ethoxyphenyl)-1-(2-hydroxyethyl)thiourea]; NSAID, nonsteroidal anti-inflammatory drug; LC-MS/MS, liquid chromatography/tandem mass spectrometry; COX, cyclooxygenase; HPLC, high-performance liquid chromatography; NBT, nitroblue tetrazolium; AUC, area under the plasma concentration versus time curve.

clearly distinct from that involved in the precipitation of gastric lesions induced by NSAIDs. For example, although inhibition of COX-1 and/or COX-2 may contribute to the toxicity (Sigthorsson et al., 2002; Tanaka et al., 2002; Hotz-Behofsits et al., 2010), there are also off-target adverse effects involved (Somasundaram et al., 1997). These "topical effects" are thought to be mediated by the glucuronide conjugates of NSAIDs (and/or their oxidative metabolites), the major export form delivering the NSAIDs from the hepatobiliary system to the small intestinal lumen. Here, the conjugates are enzymatically cleaved by β-glucuronidases, and the aglycone is reabsorbed (Seitz and Boelsterli, 1998; Treinen-Moslen and Kanz, 2006). Locally high intracellular levels of NSAIDs, combined with COX inhibition, may then initiate a cascade of events leading to epithelial damage and entailing an inflammatory response, which is triggered by increases in the permeability of the gut mucosa. This allows intestinal bacterial lipopolysaccharide to activate Toll-like receptor 4 on macrophages, leading to tumor necrosis factor-mediated cell injury and secondary activation of the innate immune system and recruitment of inflammatory cells to the site of injury (Watanabe et al., 2008). Previous studies have aimed at targeting one or more of these pathways in an attempt to develop cytoprotective strategies against NSAID enteropathy (Watanabe et al., 2008; Ramirez-Alcantara et al., 2009; LoGuidice et al., 2010; Yamada et al., 2011). Here, we sought to target a mechanism that would provide effective protection against NSAID enteropathy upstream of these primary and secondary events by limiting the initial exposure of the intestinal mucosa to the drug.

This novel strategy is based on a characteristic pharmacokinetic feature of diclofenac (DCF) and other carboxylic acid-containing NSAIDs. A portion of the hepatic diclofenac pool is conjugated with glucuronic acid to form a water-soluble 1-β-*O*-acyl glucuronide. This acyl glucuronide (AG) is readily excreted across the hepatocanalicular membrane via ATP-binding cassette sub-family C member 2 (ABCC; MRP2) into the biliary tree (Seitz and Boelsterli, 1998) and delivered to more distal sites, i.e., the jejunum and ileum (Boelsterli and Ramirez-Alcantara, 2011). During this transport, a portion of the AG is converted to iso-glucuronides by spontaneous acyl migration of the aglycone along the sugar ring (Dickinson and King, 2001). Diclofenac AG (but not the iso-glucuronides) can be cleaved by bacterial β-glucuronidase in the lumen of the small bowel. The released DCF is then taken up by enterocytes and undergoes enterohepatic circulation, thus re-exposing the mucosa repeatedly. We hypothesized that the intraluminal release of the parent drug by bacterial β-glucuronidase could be a key factor in the initiation of NSAID enteropathy; hence, highly selective inhibition of bacterial β-glucuronidase would protect against intestinal injury.

Because a normal gut flora is important for maintaining a normal health status, the targeted inhibition of a bacterial enzyme without killing the bacteria altogether may prove to be a promising approach. Recently, a number of selective bacterial β-glucuronidase inhibitors were shown to be highly efficacious against the enzyme target in aerobic and anaerobic bacteria without killing the bacteria or inhibiting the orthologous mammalian enzyme (Wallace et al., 2010). These inhibitors afforded protection against the intestinal toxicity associated with the anticancer drug CPT-11 (irinotecan) through inhibition of the release of the toxic aglycone in the gut. Here, we aimed at using one of these bacteria-selective inhibitors, Inhibitor-1 (Inh-1; Fig. 1A), to evaluate its potentially protective effects against the enteric toxicity induced by DCF in an established mouse model of NSAID enteropathy.

Materials and Methods

Chemicals. Diclofenac was obtained from Sigma-Aldrich (St. Louis, MO). Inhibitor-1 [1-((6,8-dimethyl-2-oxo-1,2-dihydroquinolin-3-yl)methyl)-3-(4-ethoxyphenyl)-1-(2-hydroxyethyl)thiourea], as described previously (Wallace et al., 2010), was synthesized in house. Diclofenac-1-β-*O*-acyl glucuronide was obtained from LC Scientific, Inc. (Concord, Ontario, Canada). All chemicals were of the highest grade available.

***Escherichia coli* β-Glucuronidase Enzyme Inhibition Studies with Inh-1.** Expression and purification of *E. coli* β-glucuronidase was conducted as described previously (Wallace et al., 2010). DCF-AG assays were performed at 50 μl total volume in 96-well assay plates (Corning Life Sciences, Lowell, MA). Reactions consisted of the following: 25 μl of assay buffer (2% DMSO, 100 mM NaCl, and 100 mM HEPES, pH 6.8), 15 μl of substrate (DCF-AG), 5 μl of Inh-1 solution, and 5 μl of 5 nM enzyme. Each reaction was quenched with trichloroacetic acid to a final concentration of 10% trichloroacetic acid. Samples were centrifuged at 13,000g for 10 min to pellet the precipitate before sample detection. HPLC-UV detection of the DCF product was carried out in a similar protocol as reported previously (Seitz et al., 1998) using a Phenomenex Luna 5 μm C18(2) reverse-phased HPLC column. The AUC for the peak corresponding to the product DCF was calculated for each inhibitor concentration.

Animals and Treatment. Male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were acclimatized for 3 weeks before the experiment and were 10 to 12 weeks of age at the start of the experiments. The animals were kept on a 14/10-h light/dark cycle. They received mouse chow (Teklad Global Rodent Diet; Harlan Laboratories, Boston, MA) and water ad libitum. All studies were approved by the Institutional Animal Care and Use Committee of the University of Connecticut. Diclofenac was dissolved in 10% (in phosphate-buffered saline) Solutol HS-15 solution and administered intraperitoneally in a volume of 10 μl/g b.wt. The ulcerogenic dose (60 mg/kg) was chosen based on a previous dose-response analysis (Ramirez-Alcantara et al., 2009). Also, we have previously shown in rats that the extent of small intestinal injury was qualitatively and quantitatively similar for both peroral or intraperitoneal routes of administration, because the development of enteropathy critically depends on portal delivery of DCF to the liver, followed by hepatobiliary export of DCF conjugates (Seitz and Boelsterli, 1998). All animals were treated at 5 h before the start of the dark cycle. Inhibitor-1 or vehicle (0.5% methyl cellulose) was administered by oral gavage b.i.d. (10 μg per mouse), starting 1 day before DCF administration and with the last dose given 1 h before DCF to minimize drug-drug interactions. This daily dose of Inh-1 was adopted from a previous mouse study where it has proven to be effective in inhibiting intestinal bacterial β-glucuronidase (Wallace et al., 2010). Control animals received methyl cellulose and/or Solutol HS-15.

Assessment of Intestinal Permeability In Vivo. Intestinal permeability changes were determined as described previously (Napolitano et al., 1996), with minor modifications. In brief, mice were administered FITC-dextran (4 kDa) by oral gavage (400 mg/kg, in 0.5% methyl cellulose) 3 h before blood collection by cardiac puncture. Serum was prepared and stored at −80°C until used. After dilution of the serum (1:10), fluorescence was recorded in black 96-well plates at λ = 490 nm/530 nm (excitation/emission, respectively). The fluorescence measurements were linear with respect to the concentration range, and the absolute values were determined with a standard curve.

Assessment of Small Intestinal Injury. Enteropathy was assessed and graded as described previously (Ramirez-Alcantara et al.,
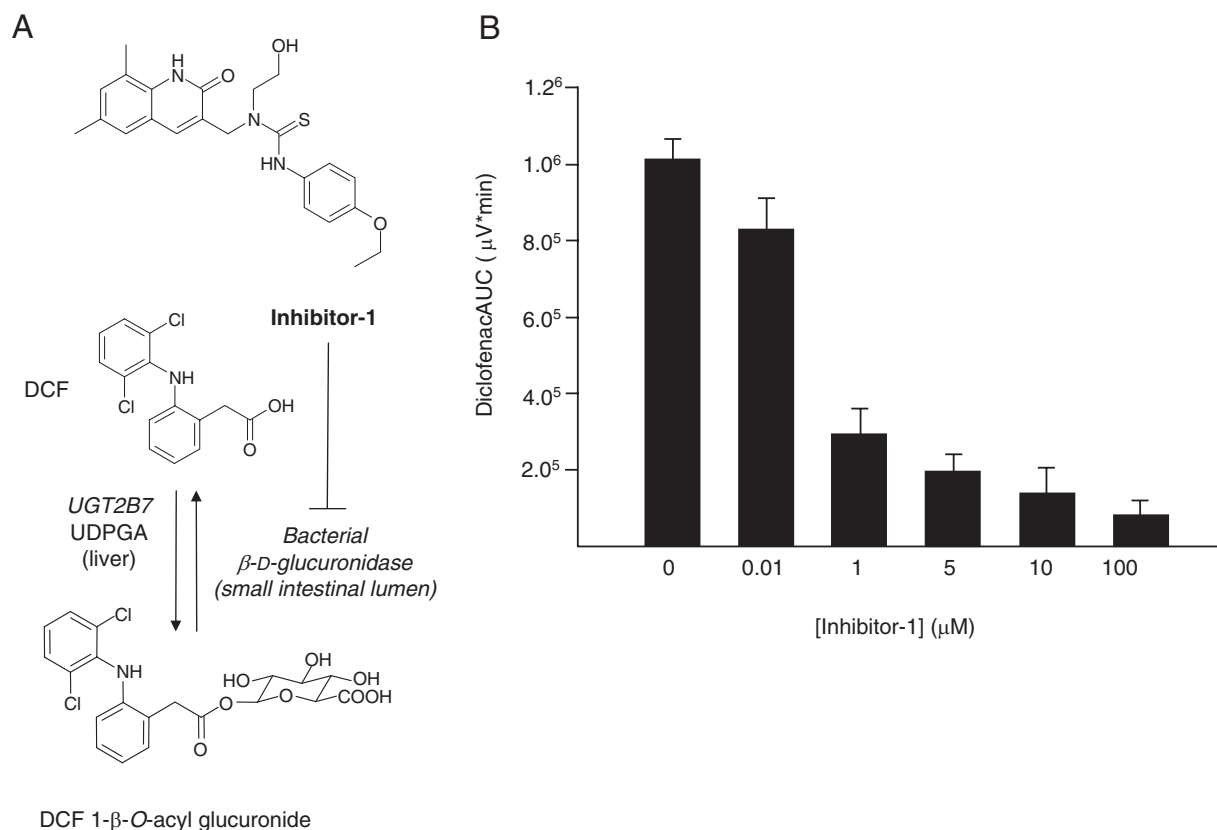
Fig. 1. Diclofenac-1-β-*O*-acyl glucuronide as a substrate for *E. coli* β-glucuronidase and inhibition of enzymatic hydrolysis by Inh-1. A, chemical structure of Inhibitor-1 and conjugation-deconjugation cycling of DCF and its inhibition by Inh-1. UGT2B7, uridine diphosphate glucuronosyl transferase 2B7; UDPGA, uridine diphosphate glucuronic acid. B, in vitro studies with purified *E. coli* β-glucuronidase and DCF-AG (4 mM) were performed as described under *Materials and Methods*. Enzymatic generation of the product, free DCF, was assessed by HPLC analysis. Inhibitor-1 caused a concentration-dependent inhibition of product formation.

2009). In brief, mice were sacrificed by CO_2 inhalation at 18 h after DCF (when the development of mucosal injury was maximal). A midline incision was made, and blood was obtained via cardiac puncture. Serum was prepared and frozen at $-80°C$ until use for analysis. The entire small intestine (from the gastroduodenal junction to the ileocecal junction) was removed and opened longitudinally along the antimesenteric side. The tissue was rinsed in ice-cold phosphate-buffered saline and incubated for 15 min in 1 mM nitroblue tetrazolium (NBT) solution containing 16 mM HEPES-NaOH, 125 mM NaCl buffer, 3.5 mM KCl, and 10 mM glucose. Next, the tissues were fixed in 10% zinc formalin for 24 h, washed, and transferred to 70% ethanol. The intestine was metrically divided into four quartiles of equal length and evaluated at $10\times$ magnification for quantitative and qualitative analysis of mucosal injury, and the lesions were assigned to the respective quartiles. The following scoring system was used: 0, no apparent lesions; 1, small erosions or ulcers (<0.1 mm); 2, medium ulcers (0.1–0.8 mm); 3, large confluent ulcers (>0.8 mm). In addition, the total area of the lesions was quantified by ImageJ software. The formalin-fixed tissue was embedded in paraffin, and 5-μm sections were stained with hematoxylin/eosin for histopathological evaluation. Serum activity of alkaline phosphatase was measured with a kinetic colorimetric kit (BioAssay Systems, Hayward, CA).

Pharmacokinetic Profiling of Diclofenac in Mice. To determine whether inhibition of bacterial β-glucuronidase might alter the disposition of DCF and its overall systemic exposure, the plasma concentration versus time curve of DCF alone was compared with that of combined Inh-1/DCF treatment. Mice were administered Inhibitor-1 (10 μg per mouse, b.i.d., for 2 days; $n = 9$) or vehicle (0.5% methyl cellulose; $n = 9$), followed by DCF (60 mg/kg i.p.). From each mouse, maximally three blood samples were serially collected by retro-orbital puncture, so that for each time point, samples were collected from three mice. Blood samples ($\sim$120 μl) were obtained at 0.16, 0.5, 1, 2, 4, 8, and 24 h after dose. Immediately after collection, plasma was prepared and stored below $-70°C$ until analysis. All samples were processed for analysis by precipitation using acetonitrile and analyzed by LC-MS/MS (Sai Advantium Pharma Ltd., Pune, India), with a lower limit of quantitation for diclofenac of 5.0 ng/ml. In brief, an ACE 3 C18 column (150 $\times$ 4.6 mm; 3.5 μm) was used; the mobile phase for gradient elution consisted of 5 mM ammonium formate in water and acetonitrile. Mass spectrometry was performed with an API 4000 (AB Sciex, Framingham, MA) equipped with a turbo ion spray source. The Noncompartmental-Analysis module in WinNonlin (version 5.2; Pharsight, Mountain View, CA) was used to assess the pharmacokinetic parameters.

Statistical Analysis. All data were expressed as mean $\pm$ S.D. If there was normal distribution, a standard analysis of variance was used, followed by Dunnett's test for multiple comparisons versus the control group. When normality failed, a Kruskal-Wallis one-way analysis of variance on ranks was used followed by Dunn's test for multiple comparison versus the control group. A P value of < 0.05 was considered statistically significant.

Results

**Inhibitor-1 Is a Potent Inhibitor of *E. coli* β-D-Glucuronidase Diclofenac 1-β-*O*-Acyl Glucuronide Cleavage.** To test our hypothesis that Inh-1 would prevent the enzymatic hydrolysis of luminal DCF-AG by enteric bacterial β-D-glucuronidase, we first determined whether DCF-AG was a substrate for bacterial β-glucuronidase in vitro. Puri-

fied *E. coli* β-glucuronidase converted DCF-AG (various concentrations) to its aglycone in vitro (Fig. 1B). Next, to assess the inhibition characteristics of Inh-1, we added increasing concentrations of the inhibitor to the incubation system containing 4 mM DCF-AG. Inhibitor-1 decreased the release of free DCF in a concentration-dependent manner, resulting in >90% inhibition at 100 μM inhibitor (Fig. 1B). The IC_{50} value was calculated to be ~164 ± 11 nM (*n* = 3 independent experiments). Previous studies had revealed that the inhibition of bacterial β-glucuronidase by Inh-1 exhibits uncompetitive characteristics (Wallace et al., 2010). These data demonstrate 1) that bacterial β-D-glucuronidase is involved in the hydrolytic cleavage of DCF-AG and 2) that Inh-1 inhibits this reaction with a high potency in the low nanomolar range.

Inhibitor-1 Protects Mice from Diclofenac-Induced Small Intestinal Ulceration. We hypothesized that if Inh-1 inhibited bacterial β-D-glucuronidase activity in vivo, then this should lead to less DCF-AG being hydrolyzed in the small intestine and to smaller amounts of released DCF interacting with the mucosa. Therefore, to determine the toxicologic consequences of inhibiting intestinal bacterial β-glucuronidase, we treated mice with a single ulcerogenic dose of DCF (60 mg/kg, i.p.) with or without pretreatment with Inh-1 (10 μg per mouse, b.i.d, for 2 days) and analyzed the extent of drug-induced enteropathy. As expected, mice receiving DCF alone developed typical NBT-positive areas of ulceration in the jejunum and ileum (Fig. 2A), whereas vehicle controls did not exhibit any apparent pathological lesions. In contrast, in mice pretreated with Inh-1, DCF caused only few isolated and small NBT-positive areas. Histopathological analysis of these lesions revealed that DCF induced typical ulcers characterized by loss of villi and an inflammatory response, involving the entire mucosa (Fig. 2B), whereas pretreatment with Inh-1 largely prevented these changes. Quantitative analysis revealed that the ulcers, which were abundant in DCF-alone-treated mice in quartiles 3 and 4, were greatly reduced both in size and number after cotreatment with Inh-1 (Fig. 3, A and B). Likewise, the DCF-associated decrease in serum alkaline phosphatase activity (an established biomarker of enteropathy) (Ramirez-Alcantara et al., 2009) was largely prevented by Inh-1 pretreatment (Fig. 3D). Furthermore, the transmucosal permeability of FITC-dextran (a high-molecular-weight, nonmetabolizable branched glucan labeled with fluorescein, which normally is poorly absorbed into the systemic circulation) was increased by almost 2-fold after DCF alone but remained at vehicle control levels after pretreatment with Inh-1 (Fig. 3C). Together, these data indicate that Inh-1 afforded significant protection from DCF-induced acute small intestinal injury in mice.

Effects of Inh-1 on DCF Pharmacokinetics. To assess whether inhibition of intestinal bacterial hydrolysis of DCF glucuronides would alter the disposition of DCF, and thus potentially impair its pharmacologic efficacy at the target tissues, we conducted a pharmacokinetic study in mice after a single administration of DCF (60 mg/kg) with and without pretreatment with Inh-1 (Fig. 4). Analysis of the DCF plasma level over time curve revealed that there was a typical biexponential decrease in plasma concentration with an apparent T_{max} of 10 min and an apparent C_{max} in the high micromolar range (Table 1). Pretreatment with Inh-1 did not significantly change these parameters, and the overall systemic exposure to DCF (AUC_{0-24h}) was not significantly different from that in DCF-alone-treated mice (exposure ratio vehicle group/Inh-1 group was 0.99). These data indicate that the amount of DCF acyl glucuronide (and possibly other glucuro-
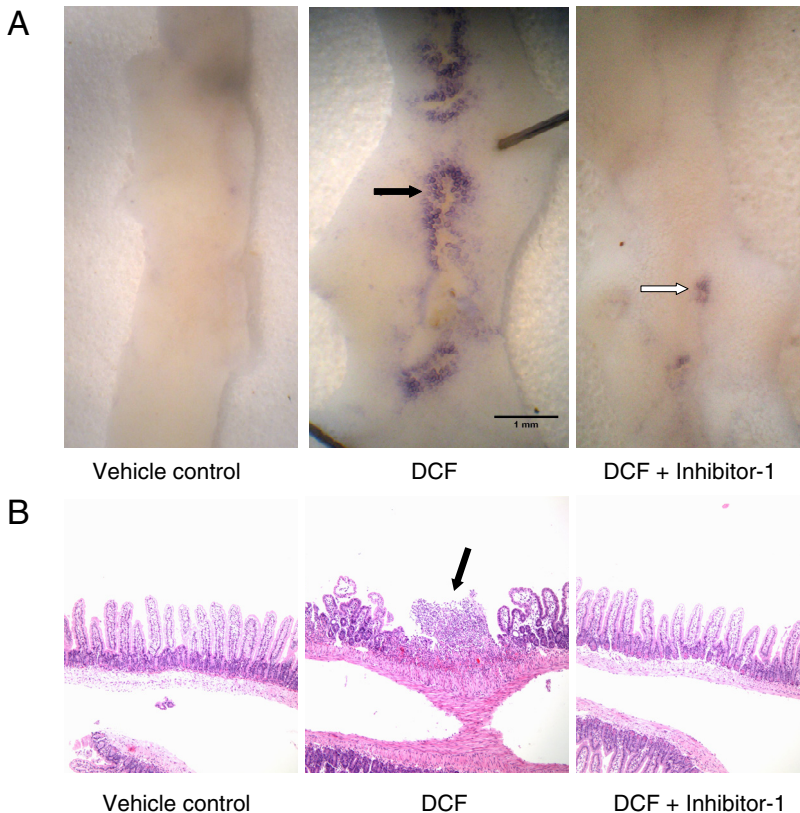
Fig. 2. Macroscopic and histopathologic assessment of mucosal damage in mouse jejunum 18 h after DCF administration with and without pretreatment with Inh-1. A, macroscopic view of the luminal side of the jejunum; note the large ulcers (arrow) in DCF-treated mice and the absence of any large ulceration in the Inh-1/DCF-cotreated mice. After Inh-1/DCF cotreatment, erosions or single small ulcers were present (white arrow) (NBT staining; 10× magnification). B, representative histopathological sections of the jejunum; note the ulcer in the DCF-treated mouse involving the entire mucosa and characterized by an inflammatory response (arrow). These lesions are not present in Inh-1/DCF-cotreated mice (hematoxylin and eosin; 40× magnification).
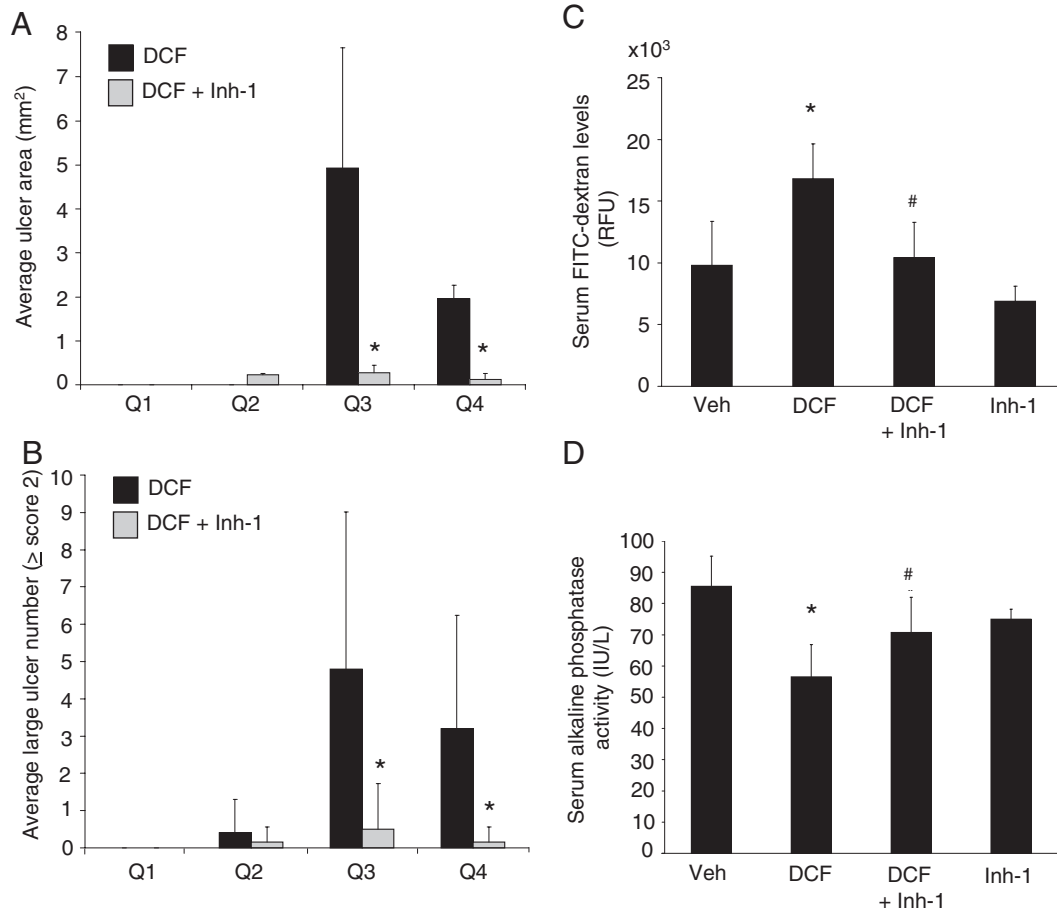
Fig. 3. Protection from diclofenac-induced small intestinal ulceration by Inh-1 pretreatment. Male C57BL/6 mice were administered a single intraperitoneal dose of DCF (60 mg/kg) or vehicle (10% Solutol HS-15) after pretreatment with Inh-1 (10 μg per mouse, p.o., b.i.d.) or vehicle (0.5% methyl cellulose) for two consecutive days. A, the average ulcer area was determined by morphometric analysis of photomicrographs using ImageJ software. The quartile numbers (Q1–Q4) are assigned to four small intestinal segments of equal length from the gastroduodenal junction to the ileocecal junction. Data are mean ± S.D. ($n = 7$ mice per group). ∗, $P < 0.05$ versus DCF alone in the respective quartile. B, average large ulcer number (score 2 or higher). Data are mean ± S.D. ($n = 7$). ∗, $P < 0.05$ versus DCF alone in the respective quartile. C, serum FITC-dextran levels as an indication of intestinal permeability. FITC-dextran was orally administered (400 mg/kg) 15 h after DCF, and blood was collected 3 h later. Data are mean ± S.D. ($n = 5$). ∗, $P < 0.05$ versus vehicle control; #, versus DCF alone. D, serum alkaline phosphatase activity 18 h after DCF. Data are mean ± S.D. ($n = 5$). ∗, $P < 0.05$ versus vehicle control; #, versus DCF alone.
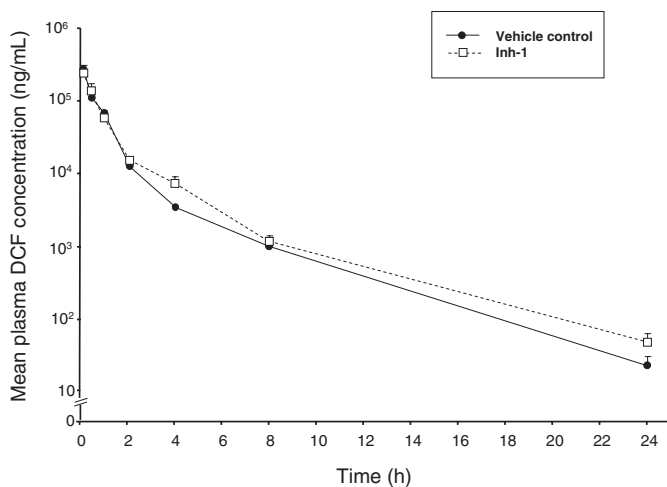
Fig. 4. Effect of Inh-1 on the pharmacokinetic profile of DCF. Male C57BL/6 mice were administered a single dose of DCF (60 mg/kg, i.p.) with or without pretreatment with Inh-1 (10 μg per mouse, b.i.d., for 2 days). Serial blood sampling was performed at the indicated time points. Diclofenac plasma concentrations were determined by LC-MS/MS as described under *Materials and Methods*. Data points are mean ± S.D. ($n = 3$ mice per time point; maximal three time points per mouse).

TABLE 1

Effects of Inh-1 on the pharmacokinetic parameters of diclofenac

Male C57BL/6 mice were pretreated with Inh-1 (10 μg p.o., per mouse) or vehicle (0.5% methyl cellulose) b.i.d. for 2 consecutive days. Diclofenac (60 mg/kg i.p.) was administered 1 h after the last dose of Inh-1. Blood was drawn after 0.16, 0.5, 1, 2, 4, 8, and 24 h after diclofenac, and plasma drug concentrations were determined by LC-MS/MS. The data are mean ± S.D. ($n = 3$ mice for each time point; maximally three serial sampling times per mouse).

Treatment	T_{max}	C_{max}	$AUC_{0-24\,h}$	$T_{1/2}$
	h	*ng/ml*	*ng/ml * h*	*h*
Diclofenac	0.16	264,868 ± 46,216	208,425	2.82
Diclofenac + Inh-1	0.16	240,115 ± 18,020	210,014	2.77

$T_{1/2}$, half-life.

nide metabolites) excreted into the bile and reaching the lower GI is sufficient to trigger mucosal injury to the small intestine, but not high enough to significantly alter the systemic exposure if its hydrolytic cleavage is prevented by the bacterial β-glucuronidase inhibitor.

Discussion

The purpose of this study was to test the hypothesis that pharmacologic inhibition of intestinal bacterial β-D-glucuron-

idase by a bacteria-specific small-molecule inhibitor would protect the small intestinal mucosa from DCF-induced ulceration. This concept was based on the hypothesis that inhibition of the catalytic release of the parent aglycone from the AG or other glucuronides would greatly reduce the high local exposure of the mucosa to the aglycone and thus alleviate toxicity. We found that one of the previously characterized selective bacterial β-D-glucuronidase inhibitors, Inh-1 (Wallace et al., 2010), indeed afforded significant protection against an ulcerogenic dose of DCF in mice. This conclusion was based on two major experimental findings. First, purified *E. coli* β-glucuronidase readily catalyzed the deconjugation of DCF-AG to DCF in vitro, confirming that DCF-AG is a substrate for bacterial β-glucuronidase. Inhibitor-1 turned out to be a highly potent inhibitor of this reaction, exhibiting an IC_{50} value in the low nanomolar range. With regard to specificity, previous studies had clearly demonstrated that Inh-1 did not inhibit the mammalian orthologs of β-glucuronidase (Wallace et al., 2010), supporting the concept that the enzyme function in the host enterocytes remained unaffected and that the bacterial form was the major target. Second, pretreatment with Inh-1 prevented the DCF-induced increases in intestinal permeability and reduced the mucosal ulceration to near-vehicle control levels as inferred from macroscopic, histopathologic, and clinical-chemical data analysis.

The importance of luminal bacteria in the development of NSAID enteropathy has been recognized for a long time; however, this has been traditionally ascribed to the initiation of an inflammatory reaction through lipopolysaccharide-mediated activation of Toll-like receptor 4 (Scarpignato, 2008; Watanabe et al., 2008). Although this is important in the propagation and amplification of the inflammatory response, we show here that the luminal bacteria play an equally crucial role in the early pathogenesis of NSAID toxicity by catalyzing the release of the parent compound from its glucuronide conjugate. The data also provide novel insights into the previous uncertainties of whether AGs, which are electrophilic species with differential reactivity toward protein targets, might be directly involved in the toxicity of carboxylic acid-containing drugs (Spahn-Langgut et al., 2007; Boelsterli and Ramirez-Alcantara, 2011). If DCF-AG were indeed the toxic species, as opposed to the free parent drug or certain oxidative metabolites, then coadministration of Inh-1 would increase the degree of enteropathy rather than protect from it. Thus, the traditional concept stating that NSAID AGs are critically involved in intestinal toxicity (Seitz and Boelsterli, 1998) can now be clarified to the following: glucuronide metabolites remain critical, but they may merely be a transport form delivering the drug from the liver to more distal sites in the GI tract, i.e., the jejunum and ileum (Boelsterli and Ramirez-Alcantara, 2011).

This revised concept may also provide a possible explanation why rodents are extremely sensitive to NSAID enteropathy, which may be related to the route of NSAID metabolite excretion. Rodents, especially rats, secrete higher levels of glucuronides into bile, because of the lower molecular weight cutoff (300–400 Da) at the canalicular plasma membrane (Klaassen and Watkins, 1984), and DCF glucuronides are clearly above this threshold. For example, DCF administration to gallbladder-cannulated mice revealed that the amount of biliary DCF-AG was 117-fold higher than that of the parent DCF and 22-fold higher than that of ring-hydroxy-

lated DCF (Lagas et al., 2010). In contrast, the canalicular export cutoff in humans is higher (~500 Da) (Bailey and Dickinson, 1996), and a larger portion of glucuronides are excreted renally rather than via the hepatobiliary route. Nevertheless, a considerable amount of DCF is excreted via bile in humans; initial studies had estimated this to be 10 to 20% (Stierlin et al., 1979), but subsequent studies had revealed that approximately 75% of total DCF clearance was in the form of glucuronides, including AGs of the major oxidative metabolite, 4′-hydroxy-DCF (Kumar et al., 2002).

If such a large proportion of an administered dose of DCF is excreted via the hepatobiliary route [42% of [^{14}C]-DCF equivalents appeared in bile for 60 min after intravenous administration of 5 mg/kg in mice (Lagas et al., 2010)], then the question may arise why inhibition of glucuronide cleavage by bacterial β-glucuronidase did not have any apparent effects on the DCF plasma level versus time curve (Fig. 4 and Table 1). A plausible reason may be that in mice the amount of DCF-AG in bile was only ~4% of the dose; the remaining conjugated metabolites (~30% of the dose) were iso-glucuronides (which are not a substrate for β-glucuronidase), glucuronides of the 4′-hydroxy or 5-hydroxy metabolites (Lagas et al., 2010), or even taurine conjugates (Sarda et al., 2012). Thus, despite the inhibition of the bacterial enzyme, the therapeutic efficacy of the NSAID probably remains intact.

Apart from bacterial β-glucuronidase, NSAID acyl glucuronides can also be cleaved to their aglycone by other enzymes. For example, it has been previously demonstrated in a guinea pig model that tissue esterases can hydrolyze intravenously administered zomepirac acyl glucuronide, because this reaction was inhibited by the nonspecific esterase inhibitor, phenylmethylsulfonyl fluoride (Smith et al., 1990). Cotreatment with phenylmethylsulfonyl fluoride decreased the futile cycling of the parent zomepirac and its glucuronide and increased the plasma clearance of zomepirac. However, these studies only addressed the role of systemic esterases; in fact, the experiments were performed in bile duct-ligated animals to prevent biliary clearance. As to intestinal enzymes, a study using rat intestinal homogenates and the NSAID diflunisal has revealed that esterases are quantitatively less important than β-glucuronidases in cleaving the acyl glucuronide (Dickinson and King, 2001). Because luminal bacterial β-glucuronidase interacts with the glucuronides before the drug is absorbed, and because specific inhibition of bacterial β-D-glucuronidase by Inh-1 greatly decreased the extent of enteropathy in this present study, it is reasonable to conclude that the enteric bacteria play a key role in the local liberation of the parent NSAID and in the subsequent initiation of enteropathy.

Intestinal epithelial cells also express β-glucuronidase activity (primarily in the endoplasmic reticulum), but the role of these host enzymes, as opposed to the bacterial enzyme, in the cleavage of NSAID glucuronides remains less clear. However, the findings that human intestinal microsomes (in contrast to liver microsomes) did not cleave a number of distinct glucuronide substrates (Oleson and Court, 2008) supports the concept that bacteria are more important in the hydrolysis of luminal glucuronides. Indeed, Inh-1 is highly specific for bacterial β-glucuronidase and has no apparent inhibitory effects on the mammalian orthologous enzyme (Wallace et al., 2010) or on intestinal carboxylesterases (M. Redinbo, unpublished data).

In addition to enzymatic cleavage, NSAID glucuronides can also undergo spontaneous, nonenzymatic hydrolysis to generate the aglycone. For example, acyl glucuronides, which are stable at acidic pH (<5), can readily undergo hydrolytic cleavage at increasing pH (Regan et al., 2010). It is noteworthy that the pH in mouse small intestine has been reported to exhibit little variation along the jejunum and ileum, ranging between 4.8 (fed state) and 5.0 to 5.2 (fasted state) (McConnell et al., 2008). Thus, the contribution of spontaneous hydrolysis in the generation of parent DCF probably plays a minor role in mice compared with the enzymatic, β-glucuronidase-catalyzed release of the free drug.

The hepatic metabolism of DCF in mice is complex, and a recent study has revealed that at least 37 distinct metabolites are generated (Sarda et al., 2012). Among these are not only acyl (ester) glucuronides but also phenol (ether) glucuronides, generated from 4′-hydroxy and 5-hydroxy DCF intermediates. These phenol glucuronides are distinct from the acyl glucuronide in their chemical stability, and they lack the reactivity toward nucleophilic targets. However, the findings that the ether glucuronides were excreted primarily in the urine of mice (Sarda et al., 2012) suggest that these metabolites may play a minor role in the enteric toxicity of DCF.

With a view to potential clinical applications of this novel concept, we note the following. First, it has been previously suggested that therapeutically altering the intestinal microflora, e.g., by administration of poorly absorbable antibiotics, might provide an enteroprotective therapy against NSAID toxicity (Scarpignato, 2008). However, this approach could have serious adverse effects in patients because a normal commensal microflora is essential. Second, strategies that have been traditionally used to protect the upper GI from NSAID ulceration, i.e., coadministration of proton pump inhibitors, do not protect the small intestine but rather can exacerbate NSAID enteropathy, as a result of a shift in the number of specific types of enteric bacteria (Wallace et al., 2011). Here, we propose an alternative strategy that includes the use of a bacteria-specific β-glucuronidase inhibitor that leaves the intestinal microbiome intact (Wallace et al., 2010).

In conclusion, we have demonstrated that pharmacologic targeting of luminal β-D-glucuronidase with a small-molecule inhibitor of the bacterial enzyme protects the GI against DCF-induced enteropathy. Because this inhibitor is highly specific for bacterial β-D-glucuronidase and does not affect mammalian β-glucuronidases or kill bacteria or mammalian cells (Wallace et al., 2010), this approach is novel and could have potential clinical implications. In view of the increased life expectancy in developed countries and the associated shifts in the demographic patterns, there will, undoubtedly, be an increased future demand for antirheumatic drugs. Furthermore, because of the known risks of the selective COX-2 inhibitors for developing cardiovascular complications, many health professionals will increasingly prescribe the more traditional NSAIDs (nonselective COX inhibitors). Therefore, the development of novel strategies to prevent NSAID-induced enteropathy is both timely and topical. Among these, an evaluation of Inh-1-type bacterial β-glucuronidase inhibitors merits further exploration as potentially potent and safe protective agents against NSAID-induced enteropathy.

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